Degradability of Phenolic Acid–Hemicellulose Esters: A Model System

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ABSTRACT

A synthetic model system containing p-coumaric and ferulic acids esterified to hemicellulose was used to study polysaccharide degradability. Oatspelts xylan was fractionated into A_{linear}, B_{linear} and $B_{branched}$ fractions prior to synthetic esterification with phenolic acids at treatment concentrations of 0, 25, 50, 75 and 100 g phenolic acid per kg hemicellulose. Concentrations of phenolic acids esterified to the hemicellulose fractions were determined by alkaline hydrolysis. In-vitro dry matter disappearance (IVDMD) and degradability of hemicellulose neutral sugars were measured after 48 h ruminal fermentations. Esterification efficiency of the phenolic acids to the hemicellulose fractions was low (0·3 to 13·9%) and greater for p-coumaric than ferulic acid (4·7 vs 3·1%, respectively). Concentration of esterified phenolic acids was negatively correlated with IVDMD for the A_{linear} and $B_{branched}$ fractions. Ferulic acid appeared to be more inhibitory to IVDMD than p-coumaric acid. Generally the degradability of the side chain sugars of the hemicellulose fractions was negatively correlated with esterified phenolic acid concentrations. Xylose degradation was only correlated with esterified ferulic acid level in the A_{linear} fraction. The in-vitro ruminal fermentations resulted in the degradation of the majority of the phenolic acid esters. Analysis of the synthetic phenolic acid–hemicellulose esters by ^{13}C NMR and FTIR was unable to prove the presence of monomeric phenolic acid esters. The presence of phenolic acid polyesters was unlikely because of the solubility of the synthetic phenolic acid–hemicellulose esters. The neutral sugar degradation data suggest that esterification of the phenolic acids was limited to sugars with primary hydroxyl groups. While this model system was useful for studying cell wall degradation, future studies must

employ model systems in which the chemical constituents being tested accurately model those found in nature.

INTRODUCTION

Forages contain ester linked phenolic acids in their cell walls, with concentrations being at least 10 times greater in grasses than in legumes (Hartley 1972; Jung *et al* 1983). The principal phenolic acids of forages, *p*-coumaric and ferulic acids, have been shown to be correlated with forage degradability (Hartley 1972; Burritt *et al* 1984; Jung and Vogel 1986). In general, *p*-coumaric acid and the *p*-coumaric:ferulic acid ratio have been found to be negatively correlated with in-vitro forage digestion (Hartley 1972; Burritt *et al* 1984). However, as described by Jung (1989), esterified *p*-coumaric acid concentrations are positively related to core lignin concentration. Therefore, it is not clear if the negative relationships with forage digestibility are due to core lignin or esterified *p*-coumaric acid. Since most of the *p*-coumaric acid appears to be esterified to the core lignin polymer rather than cell wall polysaccharides (Atsushi *et al* 1984; Azuma *et al* 1985), it may be reasonable to assume that esterified *p*-coumaric acid will not inhibit cell wall degradation independent of the core lignin polymer.

In an effort to directly determine if phenolic acids can reduce cell wall degradation, several workers have utilised model systems with free phenolic acids (Akin 1982; Jung 1985; Hartley and Akin 1989). While free phenolic acids will depress in-vitro ruminal degradation, the concentrations required to demonstrate the inhibition are several orders of magnitude greater than observed in forages, and virtually no free phenolic acids are present in forages (Jung 1989). Model systems containing *p*-coumaric and ferulic acids esterified to grass neutral detergent fibres and isolated celluloses have demonstrated reduced in-vitro fibre degradation at physiological concentrations of esterified phenolic acids (Sawai *et al* 1983; Jung and Sahlu 1986; Bohn and Fales 1989). These model phenolic acid–polysaccharide esters have been criticised by Bohn and Fales (1989) for possible departures from the true structure of forage cell walls. The objective of this study was to investigate the effect of phenolic acid esterification to various hemicellulose fractions on degradability and to examine some of the criticisms directed at this model system.

EXPERIMENTAL

Hemicellulose fractionation

Oatspelts xylan was obtained from Sigma Chemical Company* (St Louis, MO) and fractionated by a modification of the scheme of Gaillard and Bailey (1968).

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* Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee of the product by USDA and does not imply its approval to the exclusion of other products that also may be suitable.

The xylan was dissolved in anaerobic 2 M KOH. The solution was then acidified to pH 4·5 with glacial acetic acid under N_2 and allowed to precipitate overnight. The hemicellulose A_{linear} fraction was recovered by centrifugation at $17\,700 \times g$ for 30 min. Hemicellulose B was recovered from the supernate by precipitation in 4 vol 95% ethanol and centrifugation at $17\,700 \times g$ for 60 min. The hemicellulose B was then redissolved in a 26% (w/w) $CaCl_2$ solution by shaking overnight under a N_2 atmosphere. The B_{linear} fraction was precipitated by addition of an iodine solution (3% I_2 in 4% aqueous KI, w/v) and centrifugation at $17\,700 \times g$ for 30 min. The polysaccharides in the supernate ($B_{branched}$) were recovered, after addition of saturated $Na_2S_2O_3$ to neutralise the iodine solution, by precipitation in 4 vol 95% ethanol and centrifugation at $17\,700 \times g$ for 30 min. The B_{linear} pellet was redissolved in water, and saturated $Na_2S_2O_3$ was added to the solution to dissociate the iodine complex. The B_{linear} hemicelluloses were recovered by precipitation in 4 vol of 95% ethanol and centrifugation. All three hemicellulosic fractions were lyophilised.

Phenolic acid esterification

The p-coumaric and ferulic acids (predominately *trans*, Aldrich Chemical Co, Milwaukee, WI) were esterified to the A_{linear}, B_{linear} and $B_{branched}$ fractions as described by Sawai *et al* (1983) and Jung and Sahlu (1986). The chloride derivative of each phenolic acid was synthesised by refluxing with thionyl chloride in acetonitrile for 1 h. Phenolic acid chlorides in acetonitrile were added dropwise to the hemicellulose fractions suspended in acetonitrile plus 2% pyridine (v/v). Treatment concentrations were 0, 25, 50, 75 and 100 g phenolic acid per kg hemicellulose. The treated hemicelluloses were recovered by filtration and the residues were washed with acetone to remove non-esterified phenolic acids and organic solvents. The treated hemicellulose was air-dried under a hood for 48 h.

In-vitro degradation study

Six replicate samples (500 mg) of each treated hemicellulose were weighed into 50-ml screw-cap centrifuge tubes. The samples were inoculated with rumen fluid from a lucerne (*Medicago sativa* L) hay-fed Holstein cow (*Bos taurus* L) and incubated at 39°C for 48 h as described by Jung and Sahlu (1986). After fermentation the insoluble residues were recovered by centrifugation at $2000 \times g$ for 60 min and lyophilised. The supernates from two replicate tubes were lyophilised for analysis of soluble phenolic acids. All lyophilised insoluble pellets were weighed and two replicates of each treatment were dried at 100°C to determine DM content. In-vitro dry matter disappearance (IVDMD) was calculated by correcting insoluble residue weights for DM content and inoculum DM addition. Two further insoluble replicates were analysed for neutral sugar degradation, and the other two insoluble replicates were analysed for residual esterified phenolic acids.

Chemical analysis

Concentrations of esterified p-coumaric and ferulic acids in treated hemicellulose fractions and fermentation residues were determined by extraction with anaerobic 1 M NaOH (Jung and Shalita-Jones 1990). Phenolic acids were identified and

quantified by LC analysis on a Gilson Gradient Autoanalytical System (Gilson Medical Electronics, Inc, Middleton, WI) according to the conditions described by Jung and Shalita-Jones (1990). Confirmation of the presence and identity of esterified phenolic acids was done by GC–MS analysis of alkaline extracts of the phenolic acid–hemicellulose complexes. Phenolic acids were extracted after acidification from these alkaline solutions with methylene chloride and silylated with bis-trimethylsilyltrifluoroacetamide. Trimethylsilylated derivatives were separated on a DB-1 (J & W Scientific, Folsom, CA) fused silica capillary column (60 m × 0·25 mm, 1 μm film thickness) using a temperature programme of 50 to 250°C (10°C min^{-1}) with a split ratio of 50:1. Eluting compounds were detected with an HP 5970 mass selective detector (Hewlett-Packard, Palo Alto, CA) operated in the total ion scan mode. Proton and carbon-13 nuclear magnetic resonance (NMR) and Fourier transform infra-red (FTIR) spectroscopy were used to attempt identification of the ester linkage of phenolic acids to polysaccharide. The FTIR spectra were run as KBr disks on a Nicolet FT7199 FTIR spectrometer (Nicolet Instrument Corp, Madison, WI). The NMR spectra were run in DMSO-d$_6$ on a Bruker AM-360 wide bore instrument (Bruker Instruments, Inc. Billerica, MA). Composite pulse proton decoupled carbon-13 and DEPT spectra were obtained under both fast-relaxation (rapid pulsing, repetition time 0·6 s, 70° pulses) and slow-relaxation (repetition time 3 s, 35° pulses) conditions.

The neutral sugar content of treated hemicellulose fractions and fermentation residues were determined by the method of Theander and Westerlund (1986). Neutral sugars were quantified by HPLC (Jung and Russelle 1991) on a Dionex BioLC (Dionex, Inc, Sunnyvale, CA). The ash-corrected residue after hydrolysis of the hemicelluloses was defined as the Klason lignin fraction.

Statistical analysis

The data for IVDMD, neutral sugar degradation and phenolic acid ester recovery were analysed separately for each hemicellulose fraction as a 2 × 5 factorial design. Means comparisons were done by the F-protected least significant difference method. Correlation analyses of in-vitro hemicellulose degradation with esterified phenolic acid concentration were done using the least square means for the treatments. All calculations were done using PC-SAS (SAS 1985).

RESULTS AND DISCUSSION

Commercial oatspelts xylan was fractionated in to three subclasses based on solubility in KOH and the ability to form I$_2$ complexes as described by Gaillard and Bailey (1968). Although three separate polysaccharide fractions were isolated, the neutral sugar composition of each was similar (Table 1). This may indicate similar polysaccharides with differing degrees of polymerisation or differences in the spatial arrangement of substitutions upon the xylan backbone. The difference in neutral sugar composition of A$_{linear}$ and B$_{branched}$ may indicate an increased complexity in the branching pattern of the substituents on the xylan backbone of B$_{branched}$ as described by Gaillard (1965).

TABLE 1

Composition of hemicellulose fractions from oatspelts xylan fractionation

Fraction	Neutral sugars (g kg^{-1} DM)	Klason lignin (g kg^{-1} DM)	Neutral sugar components (mol per 100 mol)[a]					
			Fuc	Rha	Ara	Gal	Glc	Xyl
A$_{linear}$	665	58	0·1	0	16·6	1·3	1·1	80·8
B$_{linear}$	847	64	0·1	0	14·2	1·9	1·4	82·5
B$_{branched}$	882	51	0·2	0·1	13·7	4·9	1·1	79·9

[a] Fucose (Fuc); rhamnose (Rha); arabinose (Ara); galactose (Gal); glucose (Glc); xylose (Xyl).

TABLE 2

Concentrations of p-coumaric (PCA) and ferulic (FA) acids esterified to hemicellulose fractions after synthetic esterification

Fraction	Phenolic compound	Treatment (g kg^{-1} DM)					Correlation coefficient[a]
		0	25	50	75	100	
A$_{linear}$	PCA	0·01	0·12	0·33	1·28	1·15	0·92
	FA	0	0·07	0·10	0·22	0·22	0·96
B$_{linear}$	PCA	0	3·47	4·59	5·36	6·03	0·93
	FA	0	2·83	2·66	3·10	2·29	NS
B$_{branched}$	PCA	0·05	1·98	1·51	2·38	2·60	0·86
	FA	0	1·65	1·80	1·70	1·04	NS

[a] Correlation of treatment concentration with measured esterified phenolic acid concentration. NS (non-significant, $P > 0.10$).

Bohn and Fales (1989) pointed out that the esterification procedure utilised to create these phenolic acid–polysaccharide esters was non-specific for which neutral sugar or which OH group on a sugar was involved in the esterification. However, because xylose does not possess a primary hydroxyl group, esterification of the phenolic acids to the side chain sugar units, which all have primary hydroxyl groups, is favoured (Buehler and Pearson 1970). The neutral sugar degradation data discussed later also support the conclusion that most phenolic acid esterification is limited to non-xylose sugars.

Concentrations of p-coumaric and ferulic acids esterified to the hemicellulose fractions are given in Table 2. As seen previously, concentrations were of similar magnitude to those found in forages (Hartley 1972; Jung and Shalita-Jones 1990). Esterification efficiency was low (0·3–13·9%) for both phenolic acids and all hemicellulose fractions. The observed variation in esterified phenolic acid concentrations among the fractions was not surprising given the difference in branching patterns of the hemicelluloses. Ferulic acid esterification was consistently lower than p-coumaric acid for the hemicellulose fractions, unlike the equivalent

levels seen for esterification to cellulose (Jung and Sahlu 1986). Concentration of esterified p-coumaric acid was correlated ($P<0.10$) to treatment level for all hemicelluloses, whereas ferulic acid ester concentrations were only correlated ($P<0.10$) to treatments for A_{linear} hemicellulose (Table 2).

It has been suggested (Bohn and Fales 1989) that the phenolic acid chloride derivatives will react with themselves because of their OH group to form phenolic acid polyesters, ie depsides, which also can be esterified to the polysaccharides. If correct, this would be a poor model of the simple monomeric phenolic acid esters found in forages (Mueller-Harvey et al 1986). We were able to synthesise the ferulic acid polyester, but synthesis of the p-coumaric acid polyester was capricious, sometimes yielding an insoluble ester and other times yielding only soluble products. The ferulic and p-coumaric acid polyesters were insoluble in all solvents tested. When compared with an authentic ferulic acid–arabinose ester (5-O-[$trans$-feruloyl]-methyl-α-L-arabinofuranoside) by FTIR, the polyester of ferulic acid could be distinguished from the monomeric ferulic acid–arabinose ester ($v_{c=o}$ were 1690 cm^{-1} for the ferulic acid–arabinose ester and 1730 cm^{-1} for the ferulic acid polyester). However, the FTIR spectra of the hemicellulose fractions to which ferulic acid had been esterified did not indicate the presence of either ferulic acid monomeric esters or polyesters as no resolved peaks were evident in these regions. The treated hemicellulose fractions were completely soluble in dimethylsulphoxide, but ^{13}C NMR analysis did not detect the presence of ferulic acid monomeric esters. The ^{13}C-NMR spectra showed the typical xylan peaks for xylose internal units (δ 101·7, 72·7, 74·0, 75·4 and 63·3 for C_1 to C_5, respectively), xylose non-reducing terminal units (δ 76·4, 69·5 and 65·7 for C_3 to C_5, respectively), and xylose reducing groups (δ 97·5 for the C_1 β-anomer and 92·7 for the C_1 α-anomer) (Scalbert et al 1986). Arabinofuranose peaks were also evident (δ 107·2, 80·5, 77·8, 86·0 and 61·8 for C_1 to C_5, respectively) (Hirsch et al 1988). No evidence for any of the carbons in ferulic p-coumaric acid esters could be found. Proton NMR also did not detect either aromatic or double-bond protons. Presence of ferulic acid esters in the treated hemicellulose fractions was confirmed by alkaline hydrolysis and GC–MS analysis (silylated ferulic acid: MS (EI, 70 eV) m/Z 338 (M$^+$, 100), 323 (39), 308 (41), 293 (20), 249 (25), 73 (47)). The inability to confirm the presence of ferulic acid esters, either monomers or polyesters, by FTIR or ^{13}C NMR was probably due to the very low concentrations of phenolic acids which were esterified to the hemicelluloses. Based upon the inconsistent ability to synthesise a p-coumaric acid polyester, the complete solubility of the phenolic acid–hemicellulose esters, and general lack of correlation between treatment level and observed ester concentrations for ferulic acid, it was concluded that phenolic acid polyesters with hemicellulose were probably not formed. The possibility that cannot be excluded was that oligomeric phenolic acid esters were formed, which were soluble in dimethylsulphoxide.

Esterification of phenolic acids to hemicelluloses generally reduced ($P<0.05$) 48-h IVDMD (Table 3). For both p-coumaric and ferulic acids, esterified phenolic acid concentration was negatively correlated ($P<0.05$) with IVDMD for the A_{linear} and $B_{branched}$ fractions, but not for B_{linear} hemicellulose. Esterification of ferulic acid apparently was more inhibitory to hemicellulose degradation than p-coumaric

TABLE 3

In-vitro dry matter disappearance (IVDMD) of hemicellulose fractions after esterification with p-coumaric (PCA) or ferulic (FA) acids

Fraction	Phenolic compound	IVDMD $(g\ kg^{-1})$ Treatment $(g\ kg^{-1}\ DM)$					SEM^a	Correlation coefficient^b
		0	25	50	75	100		
A_{linear}	Control^c	543^d					5	
	PCA		516^e	525^{e*}	448^{f*}	452^{f*}		−0·98
	FA		523^e	473^f	428^g	420^g		−0·97
B_{linear}	Control	339^d					7	
	PCA		346^{d*}	322^d	279^e	295^e		NS
	FA		321^d	305^e	271^f	303^e		NS
$B_{branched}$	Control	499^d					6	
	PCA		468^{e*}	455^e	431^{f*}	447^{ef}		−0·94
	FA		437^e	444^e	407^f	432^e		−0·94

^{a} Standard error of the mean (SEM) for the fraction.
^{b} Correlations were based on measured phenolic acid concentration and done with treatment least square means. All correlations included appropriate control. NS (non-significant, $P > 0·10$).
^{c} Same control IVDMD value used for both phenolic acid treatments, within a fraction.
^{d,e,f,g} Means within a fraction and phenolic compound not sharing a common superscript are different ($P < 0·05$).
* Effects of the phenolic acids are different ($P < 0·05$) within a fraction and treatment level.

acid esters given the lower concentrations observed (Table 2) and equal, or greater, levels of IVDMD inhibition (Table 3). These reductions in degradability of esterified hemicelluloses were similar to previous data for esterified NDF and cellulose substrates (Sawai et al 1983; Jung and Sahlu 1986; Bohn and Fales 1989).

Degradability of xylose, the major hemicellulosic sugar, was not reduced ($P > 0·05$) by either phenolic acid ester in the hemicelluloses (Table 4). Across treatment levels, all of the non-xylose neutral sugars had lower ($P < 0·05$) degradabilities when p-coumaric and ferulic acids were esterified to B_{linear} hemicellulose, but only fucose and glucose degradabilities were inhibited ($P < 0·05$) in the A_{linear} and $B_{branched}$ fractions, respectively. Correlation analysis found significant negative correlations of neutral sugar degradation with measured phenolic acid ester concentration for all the side chain sugars in the A_{linear} and B_{linear} fractions (Table 5). Sugar degradability of the most highly branched hemicellulose fraction, $B_{branched}$, was correlated the least to phenolic acid concentration. Xylose degradation was only correlated ($P < 0·10$) with ferulic acid esterification of A_{linear} hemicellulose. The neutral sugar degradation data suggest most of the phenolic acid esterification was to the sugar residues in the side chains as expected. However, it was probably not confined to the arabinose units as appears to be the case in grass cell walls (Mueller-Harvey et al 1986).

Volatile fatty acid concentrations (not shown) of the fermentation supernates

TABLE 4

Degradability of neutral sugars in hemicellulose fractions after esterification with p-coumaric (PCA) and ferulic (FA) acids. Values are means across treatment levels

Fraction	Phenolic compound	Degradation ($g\ kg^{-1}$)				
		Xylose	Arabinose	Galactose	Glucose	Fucose
A_{linear}	Control	457	699	897	746	900^b
	PCA	391	635	869	697	731^{bc}
	FA	376	617	852	642	614^c
	SEM[a]	32	69	14	30	57
B_{linear}	Control	526	691^b	937^b	914^b	818^b
	PCA	481	459^c	781^c	744^c	516^c
	FA	482	494^c	797^c	754^c	551^c
	SEM	30	52	24	37	56
$B_{branched}$	Control	804	675	862	932^b	628
	PCA	796	654	850	608^c	632
	FA	773	601	828	512^c	592
	SEM	17	28	13	53	31

[a] Standard error of the mean (SEM).
[b,c] Means within a fraction not sharing a common superscript are different ($P < 0.05$).

TABLE 5

Correlation of neutral sugar degradation with concentration of esterified p-coumaric (PCA) and ferulic (FA) acids on hemicellulose fractions

Fractions	Phenolic compound	Correlation coefficient[a]				
		Xylose	Arabinose	Galactose	Glucose	Fucose
A_{linear}	PCA	NS[b]	−0·88*	−0·96***	−0·83*	−0·97**
	FA	−0·98***	−0·99***	−0·96***	−0·88*	−0·98***
B_{linear}	PCA	NS	−0·92**	−0·95**	−0·96***	−0·97**
	FA	NS	−0·88*	−0·98***	−0·96***	−0·98**
$B_{branched}$	PCA	NS	−0·89**	NS	−0·96***	NS
	FA	NS	NS	−0·84*	−0·91**	NS

[a] Correlations were done with least square means of each treatment and included appropriate control.
[b] Non-significant correlation ($P > 0.10$).
*,**,*** Correlation significant at $P < 0.10$, 0·05 and 0·01, respectively.

paralleled the IVDMD data. Molar proportions of acetate, propionate and butyrate in the fermentations were not altered ($P > 0.05$) by the presence of esterified phenolic acids. This agrees with previous reports for phenolic acid–cellulose esters (Jung and Sahlu 1986) and supports a blocking effect on polysaccharide hydrolases rather than an inhibition of microbial metabolism (Jung 1989).

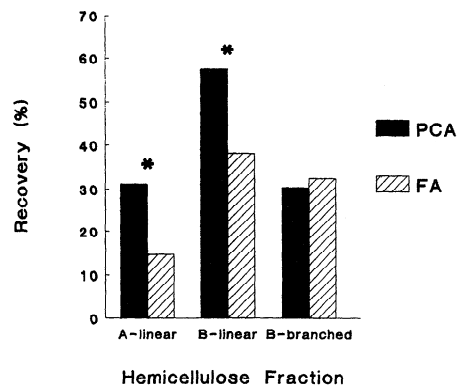Recovery of esterified phenolic acids from insoluble residues of fermented

Fig 1. Recovery of esterified p-coumaric (PCA) and ferulic (FA) acids from insoluble residues after in-vitro ruminal fermentation of hemicelluloses for 48 h. Recoveries of p-coumaric vs ferulic acids were different as indicated (*, $P < 0.05$). Standard error of the mean was 3·3%.

hemicelluloses is shown in Fig 1. Esterified p-coumaric acid was recovered to a greater ($P < 0.05$) extent from A_{linear} and B_{linear} fractions than was ferulic acid. These results agree with the greater recoveries of esterified p-coumaric than ferulic acids observed from sheep digesta after ruminal fermentation of forages (12·7–60·9 vs 4·4–34·9%, respectively) (Jung et al 1983). Concentrations of soluble p-coumaric acid were also greater than soluble ferulic acid in the rumen of sheep fed forages. Soluble esterified p-coumaric acid in the fermentation supernates accounted for 18·0, 6·9 and 0% of the total esterified p-coumaric acid in A_{linear}, B_{linear} and $B_{branched}$ hemicelluloses, respectively. No soluble ferulic acid was recovered. For both the in-vivo data (Jung et al 1983) and the current model system, recoveries of esterified phenolic acids after fermentation were greater with increasing concentrations of esterified phenolic acids.

Concern that esterification of phenolic acids to polysaccharides in this model system is confined to particle surfaces is probably justified (Bohn and Fales 1989). However, this particle size effect may be less of a problem with isolated polysaccharides, which are very fine powders, compared with neutral detergent fibre preparations from forages. In addition, use of isolated polysaccharides will reduce the opportunities for fermentation of phenolic acid–core lignin esters, as have been described for bagasse (*Saccharum officinarum* L) and bamboo (*Bambusa tulda* L) (Atsushi et al 1984; Azuma et al 1985), because of the low level of residual lignin in isolated hemicellulose compared with neutral detergent fibre. The presence of a small amount of residual core lignin in the oatspelts xylan fractions was, however, confirmed by pyrolysis–GC–MS analysis.

CONCLUSIONS

The phenolic acid–hemicellulose model system demonstrates that very low concentrations of esterified phenolic acids will reduce the degradability of polysaccharides by ruminal microorganisms. Most of this inhibition in degradation is associated with the neutral sugar components which form the side chain elements of xylans and to which phenolic acids are esterified in grasses. This model system does not appear to be as unrealistic a model as has been suggested. Efforts to identify the mechanistic relationships between cell wall matrix structure and

microbial degradation will require the continued use of model systems. Future model systems must be more carefully defined in order that the structures correspond to specific known, or potential, linkages in forage cell walls, and increasing complexity of structure should be incorporated into the model systems.

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